

The Identification of γ -L-Glutamyl-L-leucine and γ -L-Glutamyl-L-methionine in Kidney Bean Seeds (*Phaseolus vulgaris*)

CLAYTON J. MORRIS, JOHN F. THOMPSON, AND ROBERT M. ZACHARIUS

From the United States Plant, Soil, and Nutrition Laboratory, Soil and Water Conservation Research Division, United States Department of Agriculture, Ithaca, New York, and Eastern Utilization Research and Development Division, Philadelphia, Pennsylvania

(Received for publication, September 14, 1962)

During the purification of γ -glutamyl-S-methylcysteine (1), it was observed that the acidic amino acid fraction contained several other unusual ninhydrin-reactive compounds. Two of them have been isolated in crystalline form and identified as γ -L-glutamyl-L-leucine and γ -L-glutamyl-L-methionine. The identification has been established by elemental analysis, hydrolysis to the constituent amino acids, and comparison with the synthetic peptides.

γ -Glutamylleucine has been reported to occur in lima beans (*Phaseolus lunatus* L.) (2), and in onion bulbs (*Allium cepa*) (3). The isolation of γ -glutamylmethionine from onions has also been reported (3). In each case, the evidence presented for identification of the dipeptide was incomplete.

EXPERIMENTAL PROCEDURE

Preliminary Identification—One acidic amino acid fraction, from the methylcysteine isolation, contained a prominent unknown substance which moved far in *n*-butanol-acetic acid-water (9:1:2.5)¹ and phenol-water (8:3) during paper chromatography and was unstable to acid hydrolysis. A small amount of this compound was isolated by one-directional paper chromatography with Solvent 1. When this material was hydrolyzed, glutamic acid and a "leucine" were obtained in nearly equal amounts. Chromatography in tertiary butyl alcohol-benzyl alcohol-H₂O (5:2:2) (4) showed that the nonacidic amino acid was leucine and not isoleucine. It was tentatively concluded that the original compound was γ -glutamylleucine.

Another fraction, left from the isolation of γ -glutamylmethionine, when chromatographed on paper gave a spot containing a substance which reacted with iodoplatinate as well as with ninhydrin. This spot no longer appeared on hydrolysis of the fraction, but glutamic acid and a compound that chromatographed in the methionine region were formed. The identity of the methionine was confirmed by its reaction with iodoplatinate, oxidation to the sulfoxide, and chromatography in Solvent 1 and phenol. Thus, the unhydrolyzed material was, by analogy with past discoveries, probably γ -glutamylmethionine.

Quantitative paper chromatography indicated that there was enough of the leucine peptide in the fraction from the methylcysteine preparation to warrant isolation. There was not, however, enough of the methionine peptide in the fractions, so it

was necessary to use a larger batch of kidney beans for isolation of this compound.

Isolation and Identification—Leucine peptide: The following fractions containing the leucine peptide were combined. They were Fractions 120 to 200, 260 to 360, and 271 to 380 from Runs 1, 2, and 3 of the γ -glutamylmethionine isolation (1). The solvent was removed from the combined fractions with a rotary evaporator, and the solid residue was dissolved in 200 ml of Solvent 1 and applied to a paper roll (5). The paper roll was washed with Solvent 1, the flow rate being adjusted to 3 ml per minute. Fractions of 18 ml were collected, and the first ninhydrin activity appeared in Tubes 160 to 170. The fractions were examined as described in a previous paper (6), and the leucine peptide was found in 170 to 250. The peptide was contaminated by less than 5% of a ninhydrin-reactive compound. Several recrystallizations from ethanol-water eliminated the contaminant and resulted in 100 mg of pure crystalline leucine peptide.

A few milligrams of the crystalline solid were heated for 4 hours with 3 N HCl at 120°. An aliquot of the hydrolyzed material, along with standards of glutamic acid, leucine, and isoleucine, was chromatographed on paper in tertiary butanol-benzyl alcohol-water. Examination of the developed papers indicated that the peptide contained glutamic acid and leucine in approximately a 1:1 ratio. Further comparison by the buffered resin system of Moore *et al.* (7) and an amino acid analyzer confirmed that the peptide was made up of a 1:1 ratio of glutamic acid and leucine (102:103), and that the ninhydrin activity of these two compounds accounted for the whole of the peptide. The method of Connell *et al.* (8) was used for a quantitative determination of the ninhydrin activity of the unhydrolyzed peptide. The results indicated that the isolated compound could only be a dipeptide. The α -amino acid test of Kalyankar and Snell (9) was employed as previously reported (6). A positive test for an α -amino acid showed that the peptide was a γ -glutamyl peptide. Elemental analysis (see Table I) gave satisfactory results and indicated that the compound was pure.

Methionine peptide: To isolate the methionine peptide, 40 kg of red kidney beans were pulverized and thoroughly extracted with 50 to 70% ethanol. The clear amino acid extract was purified of salts and sugars by use of Dowex 50 (10).² The

¹ Henceforth, this will be referred to as Solvent 1.

² The use of trade names does not imply endorsement by the

purified extract was put on a 5.8- × 120-cm column of Dowex 1-acetate.² Thorough washing with distilled water removed all basic amino acids and most of the neutral amino acids. The aromatic amino acids, which were more tightly bound to the resin than the rest of the neutral amino acids, appeared partly in the water wash. The remainder were present in the first acid eluate. The Dowex 1 column,² which was kept in the cold room at 5°, was washed with 0.15 N acetic acid, and 20-ml samples were collected. The methionine peptide was in Tubes 1721 to 2620. These fractions were contaminated to the extent of approximately 80 to 90% with γ -glutamylmethyleysteine and other amino compounds. The majority (approximately 90%) of the methyleysteine peptide was removed by crystallization from ethanol-water. An examination of the mother liquor by paper chromatography, with the use of Solvent 1, indicated that it would be possible to separate the methionine peptide by this method. The dried material was accordingly dissolved in 200 ml of Solvent 1, transferred to the paper roll, and chromatographed. Examination of the fractions (6) from the eluate revealed a partial separation of the methionine peptide. By using only the material from the center of the peak, it was possible to obtain a chromatographically pure compound. Crystallization from acetone-water resulted in 120 mg of white plates. Elemental analysis (Table I) indicated that the isolated material was pure.

Hydrolysis and quantitative estimation by paper and ion exchange techniques confirmed that this peptide consisted of glutamic acid and methionine in a 1:1 ratio (95:101). As with the leucine compound, it was determined that this was a γ -glutamyl dipeptide of methionine.

Synthesis of γ -L-Glutamyl-L-leucine—The synthesis of this compound was exactly analogous to those previously reported by this laboratory (6, 11). In this case, 5.10 g (0.01 mole) of L-leucine benzylester-*p*-tosylate were treated with 3.86 g of α -benzyl-N-carbobenzyloxy-L-glutamic acid to produce the peptide ester. Reduction with hydrogen over palladium black, purification with Dowex 1-acetate,² and crystallization from acetone-water resulted in a 30% yield of chromatographically pure compound.

Synthesis of γ -L-Glutamyl-L-methionine—The syntheses of methionine peptides are difficult because the sulfur of the thioether poisons hydrogenation catalysts used to remove protecting groups. Iselin (12) has reviewed the problems and published a new method involving the formation of esters of methionine sulfoxide. This method and the one of Sachs and Brand (13) have been combined for the synthesis of γ -glutamylmethionine.

L-Methionine-Methyl Ester-Hydrochloride—L-Methionine (0.1 mole) was esterified with 50 ml of 2.5 N ethanolic HCl for 24 hours at 25°. The solvent was removed in a rotary evaporator, and the solid was extracted twice with ether. After being dried under vacuum over NaOH, the solid was crystallized from methanol-ethyl acetate to yield 1.5 g of the methyl ester hydrochloride.

L-Methionine Sulfoxide-Methyl Ester-Hydrochloride—The methionine methyl ester hydrochloride (1.5 g) was dissolved in 20 ml of methanol, and 1.2 ml of 30% H₂O₂ were added. The reaction mixture was left for 2 hours at room temperature. The solvent was removed at 30° with a rotary evaporator, and the

TABLE I
Elemental analysis

	γ -Glutamylmethionine			γ -Glutamylleucine		
	% Theory	Isolated	Synthetic	% Theory	Isolated	Synthetic
Carbon.....	43.15	42.9	43.0	50.57	50.6	49.8
Hydrogen....	6.52	6.36	6.34	7.76	7.20	7.13
Nitrogen.....	10.07	10.2	10.1	10.75	10.8	10.0
Sulfur.....	11.52	11.5	11.3			
$[\alpha]_D^{20}$		^a	-9.75 ^b		-18.2 ^c	-17.9 ^c
Melting points.....		202-205 ^d	203-205 ^d		180-185 ^d	180-185 ^d
<i>R_F</i> Solvent 1..		0.40	0.40		0.50	0.50
Phenol-H ₂ O...		0.70	0.70		0.80	0.80

^a Insufficient material was available to measure optical rotation.

^b *c* = 2.

^c *c* = 4.

^d With decomposition.

sulfoxide was crystallized from methanol-ethyl acetate (yield 1.54 g).

Peptide Ester— α -Benzyl-N-carbobenzyloxy-L-glutamic acid (1.85 g) (0.005 mole) was treated with 1.4 g of the L-methionine sulfoxide-methylester hydrochloride as previously described (6, 11). The peptide ester was recrystallized once from ethyl acetate-petroleum ether.

Hydrolysis of Ester—The solid ester was dissolved in 25 ml of concentrated HCl and placed in a water bath at 40°. After 1 hour the liquid was removed in a rotary evaporator at 30° under high vacuum (5 to 10 minutes). The flask containing the peptide sulfoxide was then placed in a vacuum desiccator over solid NaOH at approximately 0.1 mm pressure for 1 hour.

Reduction of Sulfoxide—The oxidized peptide was dissolved in 5 ml of water and treated with 5 ml of freshly distilled thioglycolic acid. The flask was flushed with nitrogen for several minutes, stoppered, and placed in a water bath at 50° for 20 hours. The thioglycolic acid was removed by pouring the solution through a column of Dowex 50-H⁺ (2 × 20 cm) at 5° and washing with 200 ml of cold, deionized water. The peptide was removed from the resin by elution with cold 2 N NH₄OH. When the effluent became alkaline, the column was washed with cold, deionized water until a negative ninhydrin test was obtained. The eluate was taken to dryness on a rotary evaporator at 35°. Two-directional paper chromatography showed that the peptide was contaminated with several ninhydrin-active compounds; namely, glutamic acid, methionine, methionine sulfoxide, and a small amount of the sulfoxide of γ -glutamylmethionine.

Final Purification—A solution of the synthetic γ -glutamylmethionine and contaminating materials was percolated through a column of Dowex 1-acetate² (2 × 30 cm). The two acidic peptides and glutamic acid, which were absorbed on the resin, were eluted with several hundred milliliters of cold 2 N acetic acid. The acid was removed by distillation at 35° and the solid residue dissolved in 250 ml of Solvent 1. The solution was placed on the paper roll, eluted with Solvent 1 as before, and 17-ml fractions were collected. The peptide was in Fractions 181 to 360 and was not contaminated with other ninhydrin-positive compounds. After two recrystallizations from ace-

United States Department of Agriculture, but are used only as a convenient means of designating the type of material employed.

tone-water, 350 mg of pure γ -glutamylmethionine were obtained.

Comparison of Synthetic and Isolated Peptides—The infrared absorption spectra of isolated and synthetic γ -glutamylleucine were identical as were those of isolated and synthetic γ -glutamylmethionine. Comparisons of elemental analysis, optical rotation, melting point, etc., are shown in Table I and indicate good agreement with theory and between synthetic and isolated material.

DISCUSSION

The nitrogen of γ -glutamylleucine comprised 1.41%, and of γ -glutamylmethionine, 0.48% of the nonprotein nitrogen of kidney bean seeds. This is far less than the content of γ -glutamyl-S-methylcysteine, which is responsible for 34% of the nonprotein nitrogen. The uncombined leucine was 8% of that in the peptide form, and the ratio of uncombined methionine to its peptide was approximately the same. Hydrolysis of the nonprotein nitrogen fraction of the kidney bean with 6 N HCl resulted in an increase in leucine and methionine equivalent to the amount of each in the respective peptides. This would indicate that there were no other peptides of these two amino acids present in any appreciable amounts.

These dipeptides are presumably formed from glutathione and the appropriate amino acids in the presence of a γ -glutamyl transferase (14). Methylcysteine, leucine, and methionine are all active γ -glutamyl group acceptors with the kidney bean enzyme.

Eleven γ -glutamyl peptides have been found in plants within the past decade. Bulbs and legume seeds have been particularly prominent sources. Aside from kidney beans and lima beans, soybeans (6) also contain γ -glutamyl peptides (γ -glutamyltyrosine and γ -glutamylphenylalanine). Virtanen and Mattila (15) and Suzuki *et al.* (16) have found seven dipeptides in onion and garlic bulbs. Iris bulbs (*Iris tingitana*) contain peptides of two β -amino acids (11, 17).

The recent discoveries of γ -glutamyl peptides of β -alanine (18) in honesty seeds (*Lunaria annua* L.) and of hypoglycin A (19)

in ackee fruit (*Blighia sapida*) possibly indicate a wide taxonomic distribution of these dipeptides.

SUMMARY

γ -L-Glutamyl-L-methionine and γ -L-glutamyl-L-leucine have been isolated from red kidney beans. They were obtained in pure form after ion exchange and paper chromatography.

The two dipeptides were synthesized and compared to the isolated ones with respect to elemental analysis, melting points, chromatography, optical rotation, and infrared absorption spectra.

REFERENCES

1. ZACHARIUS, R. M., MORRIS, C. J., AND THOMPSON, J. F., *Arch. Biochem. Biophys.*, **80**, 199 (1959).
2. RINDERKNECHT, H., THOMAS, D., AND ASLIN, S., *Helv. Chim. Acta*, **41**, 1 (1958).
3. VIRTANEN, A. I., AND MATIKKALA, E. J., *Suomen Kemistilehti*, **34**, 53 (1961).
4. THOMPSON, J. F., AND MORRIS, C. J., *Anal. Chem.*, **31**, 1031 (1959).
5. HAGDAHL, L., AND DANIELSON, C. E., *Nature*, **174**, 1062 (1954).
6. MORRIS, C. J., AND THOMPSON, J. F., *Biochemistry*, **1**, 706 (1962).
7. MOORE, S., SPACKMAN, D., AND STEIN, W. H., *Anal. Chem.*, **30**, 1185 (1958).
8. CONNELL, G. E., DIXON, G. H., AND HANES, C. S., *Can. J. Biochem. Physiol.*, **33**, 416 (1955).
9. KALYANKAR, G. D., AND SNELL, E. E., *Nature*, **180**, 1069 (1957).
10. THOMPSON, J. F., MORRIS, C. J., AND GERING, R. K., *Anal. Chem.*, **31**, 1028 (1959).
11. MORRIS, C. J., THOMPSON, J. F., ASEN, S., AND IRREVERRE, F., *J. Biol. Chem.*, **237**, 2180 (1962).
12. ISELIN, B., *Helv. Chim. Acta*, **44**, 61 (1961).
13. SACHS, H., AND BRAND, E., *J. Am. Chem. Soc.*, **75**, 4608 (1953).
14. THOMPSON, J. F., TURNER, D. H., AND GERING, R. K., *Federation Proc.*, **21**, 5 (1962).
15. VIRTANEN, A. I., AND MATTILA, I., *Suomen Kemistilehti*, **34**, 44 (1961).
16. SUZUKI, T., SUGII, M., AND KAKIMOTO, T., *Chem. and Pharm. Bull.*, (Japan), **9**, 77 (1961).
17. MORRIS, C. J., THOMPSON, J. F., ASEN, S., AND IRREVERRE, F., *J. Biol. Chem.*, **236**, 1181 (1961).
18. OLESEN-LARSEN, P., *Acta Chem. Scand.*, **16**, 1511 (1962).
19. HASSALL, C. H., AND JOHN, D. D., *J. Chem. Soc.*, 4112 (1960).